Specific Biomarkers Are Associated with Docetaxel- and Gemcitabine-Resistant NSCLC Cell Lines[1]

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Abstract

Five-year survival rate for lung cancer is limited to 10% to 15%. Therefore, the identification of novel therapeutic prognostic factors is an urgent requirement. The aim of this study is thus to highlight specific biomarkers in chemo-resistant non–small cell lung cancer cell lines. Therefore, we checked—in the control condition as well as after short-term pharmacological treatment with either docetaxel or gemcitabine—the expression of genes such as tumor suppressor genes (*CDKN2A*, *DAPK*, *FHIT*, *GSTP1*, *MGMT*, *RARβ2*, *RASSF1A*, and *TIMP3*), genes associated with drug resistance (*BRCA1*, *COX2*, *ERCC1*, *IGFBP3*, *RRM1*, and *TUBB3*), and stemness-related genes (*CD133*, *OCT4*, and *SLUG*) in two cellular models of squamous carcinoma (CAEP) and adenocarcinoma (RAL) of the lung originally established. Their promoter methylation profile was also evaluated. Drug-related genes were upregulated. Cisplatin resistance matched with high levels of *BRCA1* and *ERCC1* in both cell lines; docetaxel sensitivity of CAEP cells was associated to levels of *TUBB3* lower than RAL cells. Although CAEP cells were more sensitive to gemcitabine, both cell lines showed high levels of *RRM1*. Stemness-related genes were downregulated in the control condition but became upregulated in docetaxel-resistant cells, indicating the selection of a population with stemness features. We did not find an unequivocal correspondence between gene expression and respective DNA promoter methylation status, suggesting the involvement of additional mechanisms of gene expression regulation. These results highlight specific biomarkers consistent with the different responses of the two cell lines to standard pharmacological treatments and indicate specific molecular traits for their chemoresistance.

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Introduction

Non–small cell lung cancer (NSCLC) represents a common cause of tumor death in industrialized countries where, despite a significant improvement in diagnostics, surgery, and chemotherapy, the overall 5-year survival rate is in fact limited to about 10% to 15% [1,2]. The recommended treatment for these tumors is surgery, whereas radiotherapy and chemotherapy are used for the treatment of un-resectable or locally advanced tumors and as palliative therapies for metastatic tumors [3]. In tumors without *EGFR* mutations, platinum compounds, taxanes and gemcitabine—alone or in combination—are the traditional drugs used in standard chemotherapy, even though tumor resistance to these treatments is common [4].

Chemoresistance can arise from different mechanisms, such as reduced drug uptake, increased drug efflux, drug detoxification, DNA repair, or defective apoptosis, depending on the specific drug target. In this respect, several studies highlight relationships among specific gene expression, therapeutic response and tumor progression, and major classes of drugs used in the treatment of NSCLC.

In particular, resistance to platinum compounds is mainly the consequence of the overexpression of DNA repair genes, such as *ERCC1*

and *BRCA1* [5–7]. During taxane treatment of NSCLC, *BRCA1* expression represents a marker of drug sensitivity [8,9]. However, a high expression level of *TUBB3*, encoding for a member of the β-tubulin protein family, is associated with taxane resistance in different tumors, including NSCLCs [5–7]. Conversely, high levels of *FHIT* expression were shown together with significant apoptosis in *in vitro* experiments [10]. NSCLC therapy may also include the use of gemcitabine, whose intracellular active forms interfere with DNA synthesis through competition with deoxycytidine and inhibition of ribonucleotide reductase. Therefore, high levels of expression of the subunit 1 of ribonucleotide reductase (*RRM1*) are associated with increased gemcitabine resistance in NSCLC cell lines [11].

Promoter methylation is an epigenetic event usually associated with gene silencing, implicated in the development and differentiation and frequently deregulated in cancer [12,13]. Several studies have identified a pattern of promoter methylation and a corresponding gene expression profile consistent with tumor progression and response to drug treatment either in NSCLC patients or in cell lines. Several tumor suppressor genes (TSGs) display a hypermethylated promoter in lung cancer, including *CDKN2A*, *RASSF1A*, *RARβ2*, *MGMT*, *GSTP1*, *DAPK*, *TIMP3*, and *FHIT* [14–17]. Aberrant methylation of *RASSF1A*, *DAPK*, *CDKN2A*, and *FHIT* promoters was associated with shorter overall survival [18–21].

Evaluating gene expression and promoter methylation of specific genes significant for monitoring NSCLC progression might thus identify specific biomarkers that could predict disease recurrence and help in keeping watch over therapeutic response.

In the present work, to explore this issue, we used an *in vitro* model based on two NSCLC cell lines, representative of either a squamous carcinoma (CAEP) or an adenocarcinoma (RAL) of the lung, established and studied at the Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori at Meldola [22–25]. In these cells, we evaluated the expression of genes of interest in lung cancer disease, such as a set of TSGs (*CDKN2A*, *DAPK*, *FHIT*, *GSTP1*, *MGMT*, *RARβ2*, *RASSF1A*, and *TIMP3*) found to be implicated in lung cancer progression and therapeutic response [19,26–32], and a set of genes associated with specific drug resistance (*BRCA1*, *COX2*, *ERCC1*, *IGFBP3*, *RRM1*, and *TUBB3*) [3–5,33–36]. In addition, a set of genes expressed in cells with stemness features (*CD133*, *OCT4*, and *SLUG*) [37–43] was also monitored, as increasing evidence points at small cancer cell populations, defined as cancer stem cells, sustaining NSCLC resistance to antitumor agents [39,44,45] and accounting for tumor recurrence [46].

To investigate the involvement of our gene panel in the process of resistance to chemotherapy, we carried out this analysis in the basal control condition as well as after short-term pharmacological treatment with standard chemotherapeutic drugs.

Promoter methylation was also studied to evaluate how the expression profile of these genes might be consistent with this epigenetic modification.

Materials and Methods

Cell Culture

The study was performed on two *EGFR* wild-type NSCLC cell lines, CAEP and RAL, derived from a squamous carcinoma and an adenocarcinoma of the lung, respectively, established and characterized in our laboratory [22–25]. Cell lines were grown in Dulbecco's modified Eagle's medium/HAM F12 (1:1), supplemented with 10% FBS, 2 mM L-glutamine (PAA, Pashing, Austria), and insulin (10 mg/ml; Sigma-Aldrich, Milan, Italy) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Cells in the exponential growth phase were used for all the experiments.

Drugs and Treatment

Cisplatin (Ebewe Pharma-Sandoz, Origgio, Italy), docetaxel (Sanofi-aventis SpA, Milan, Italy), and gemcitabine (Eli Lilly Italia SpA, Sesto Fiorentino, Italy) were aliquoted, stored at –80°C, and freshly diluted in culture medium before each experiment.

For *in vitro* chemosensitivity assay, drugs were tested at scalar dilutions of 1:1, 1:10, and 1:100 of plasma peak concentrations (10 μM for cisplatin, 2 μM for docetaxel, and 40 μM for gemcitabine). To ensure that exposure times were compatible with the half-life of the drugs administered in a clinical setting [47–50], we analyzed the effects after 6, 1, and 3 hours of exposure, respectively, followed by 72 hours of culture in drug-free medium.

For gene expression and DNA methylation analysis, cells were treated at either 50% inhibiting concentration (IC_{50}) or plasma peak concentration dosage when IC_{50} was not reached. These values were 0.09 μM and 2 μM docetaxel and 1.98 μM and 40 μM gemcitabine, for, respectively, CAEP and RAL.

In Vitro *Chemosensitivity Assay*

Cell viability was evaluated with the sulforodamine B (SRB) assay according to Skehan et al. [51]. Experiments were run in octoplets, and each experiment was repeated twice. Dose-response curves were created by Excel Software and the IC_{50} values were determined from the plots.

Gene Expression Analysis

The analysis of gene expression was performed as reported by Arienti et al. [52] using a real-time reverse transcription–polymerase chain reaction (PCR) method. The amount of mRNA of each gene was normalized to the endogenous references β2-microglobulin using Gene Expression Macro Software (version 1.1; Bio-Rad Laboratories S.r.l., Segrate (MI), Italy). Commercial control RNA, derived from a normal lung, was used as calibrator (MPV total RNA; Stratagene, Agilent Technologies Italia SpA, Cernusco, Italy). Primers for mRNA amplification were designed using Beacon Designer Software (version 4; PREMIER Biosoft International, Palo Alto, CA); sequences and annealing temperatures are available upon request.

DNA Methylation Analysis

Promoter methylation was analyzed by a standard methylation-specific PCR (MSP) protocol from DNA extracted in phenol-chloroform. Briefly, 1 μg of purified DNA was subjected to bisulfite treatment (EZ DNA Methylation Kit; Zymo Research, Irvine, CA). The methylation status of the gene promoter was determined by MSP [53] and confirmed in two independent experiments. Total volume for MSP reaction was 15 μl, with 4 mM $MgCl_2$, 0.5 mM each dNTP, 0.2 μM each primer, 0.5 U of @Taq Hot Start Thermostable DNA polymerase (EuroClone, Pero, Italy), and 1 μl of bisulfite-treated DNA. Methylation of *OCT4* and *CD133* gene promoters was tested by bisulfite sequencing, cloning PCR products in pGEM-T Easy Vector System (Promega, Milan, Italy). Sequencing of PCR products was performed using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) using the Applied Biosystems 3130 Avant

Genetic Sequencer. Primer sequences and annealing temperatures are available upon request.

Statistical Analysis

Gene expression of CAEP and RAL cell lines in the basal control condition was statistically compared with two-tailed t test using GraphPad Prism (version 4.0; GraphPad Software, San Diego, CA). Data for basal control condition *versus* drug treatment expression were statistically analyzed using one-way analysis of variance with Tukey's post test (GraphPad Prism, version 4.0; GraphPad Software). Differences in gene expression were considered significant for $P < .05$.

Results

Pharmacological Treatment

Both the investigated cell lines showed high resistance to cisplatin treatment, even after treatment with the plasma peak concentration (10 μM; Figure 1*A*).

The squamous carcinoma cells (CAEP) showed less resistance to docetaxel and gemcitabine than the adenocarcinoma cells (RAL). In fact, the exposure of CAEP cells to 2 μM (the plasma peak concentration) docetaxel induced 80% cell death with an IC_{50} value of 0.09 μM (Figure 1*B*). When the CAEP cells were treated with gemcitabine, an IC_{50} of 1.98 μM was observed with an overall survival of about 36% at the highest dose tested (Figure 1*C*). Conversely, after either docetaxel or gemcitabine exposure, IC_{50} values were never reached even at the highest concentrations tested, for the adenocarcinoma cells (RAL; Figure 1, *B* and *C*).

Gene Expression Profile

Among the investigated TSGs, only the *CDKN2A* mRNA was highly upregulated in both lung cancer cell lines with respect to normal lung tissue. *MGMT* transcript levels were upregulated (five-fold) in RAL but not in CAEP cells. Although similar to normal lung tissue levels, *DAPK* and *FHIT* expression in RAL cells was significantly higher (respectively about 3-fold and 11-fold; $P < .01$) than in CAEP cells. No differences between CAEP and RAL were scored for *GSTP1*, *RARβ2*, *RASSF1A*, and *TIMP3*, although in both cell lines they were downregulated with respect to the normal lung tissue (Figure 2).

As expected, genes associated with specific drug resistance (*BRCA1*, *ERCC1*, *RRM1*, and *TUBB3*) were significantly upregulated in both cell lines with respect to the normal lung tissue. A significant difference in mRNA levels of RAL *versus* CAEP cells was scored for *COX2* ($P < .01$) and *TUBB3* ($P < .05$), respectively about 20-fold and 5-fold increases in RAL cells. The reverse was observed for *IGFBP3*: It was highly upregulated (about 100-fold) in CAEP *versus* RAL cells (Figure 2).

In both cell lines, *CD133* and *OCT4* gene products, associated with stemness features, were downregulated, whereas *SLUG* gene product was overexpressed with respect to normal lung tissue (Figure 2).

Upon treatment with docetaxel and gemcitabine, the expression of the investigated genes showed specific differences with respect to the basal control condition (Figure 3). Docetaxel treatment in CAEP cells did not modify the expression of TSGs; however, the *RRM1* transcript level was significantly decreased (42%) with respect to the basal control condition ($P < .05$) and *OCT4* expression in treated cells increased about six-fold ($P < .01$; Figure 3*A*). RAL cells treated with docetaxel, in contrast, showed a significantly decreased expression of the TSGs *DAPK*, *FHIT*, *MGMT*, and *RARβ2* (about 22%, 35%, 5%, and 30%, respectively) compared to the basal control condition ($P < .05$; Figure 3*B*). *CD133* expression was significantly decreased (20%; $P < .05$), whereas *SLUG* mRNA level increased about five-fold ($P < .05$; Figure 3*B*).

The only change in the expression level of the investigated genes induced by gemcitabine in CAEP cells occurred at the *TUBB3* gene, which was downregulated (52%) with respect to the basal control condition ($P < .05$; Figure 3*A*). In RAL cells, however, several effects were seen, i.e., gemcitabine treatment induced decreased expression of the TSGs *DAPK*, *FHIT*, *MGMT*, and *RARβ2* (about 18%, 6%, 7%, and 16%, respectively), compared to the basal control condition ($P < .05$; Figure 3*B*); only *COX2* was downregulated (8%, $P < .01$) among genes associated with specific drug resistance; and finally, the mRNA level of the *CD133* gene was decreased (16%) with respect to the basal control condition ($P < .01$; Figure 3*B*).

Promoter Methylation Analysis

TSG promoter was unmethylated for *DAPK*, *RASSF1A*, and *TIMP3* and partially or completely methylated for *GSTP1*, *MGMT*, and *RARβ2*, in both cell lines. *CDKN2A* promoter was methylated
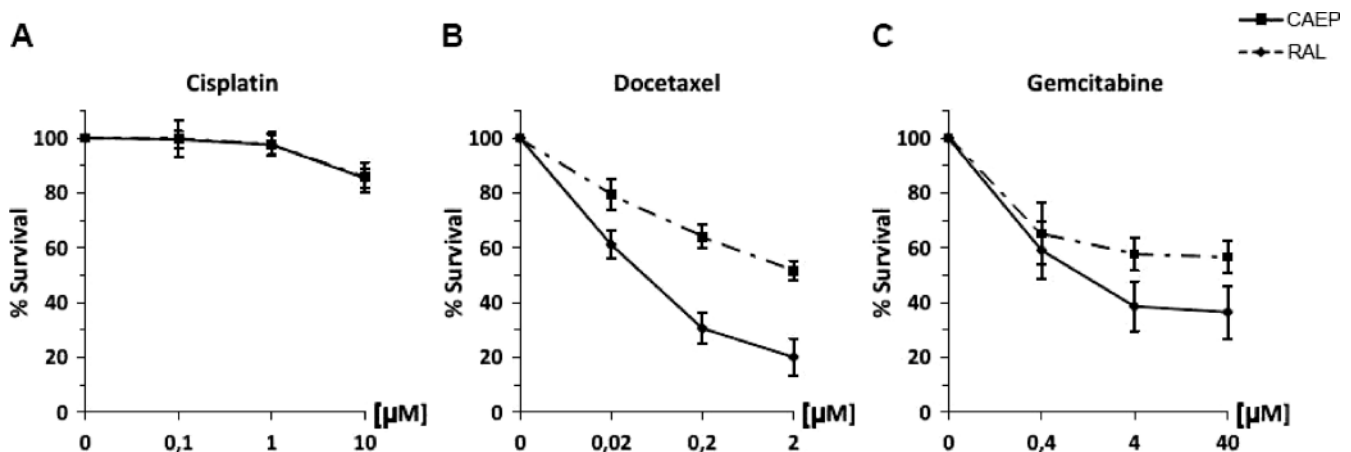

Figure 1. Cell survival of CAEP and RAL cell lines after the treatments with (A) cisplatin, (B) docetaxel, and (C) gemcitabine.
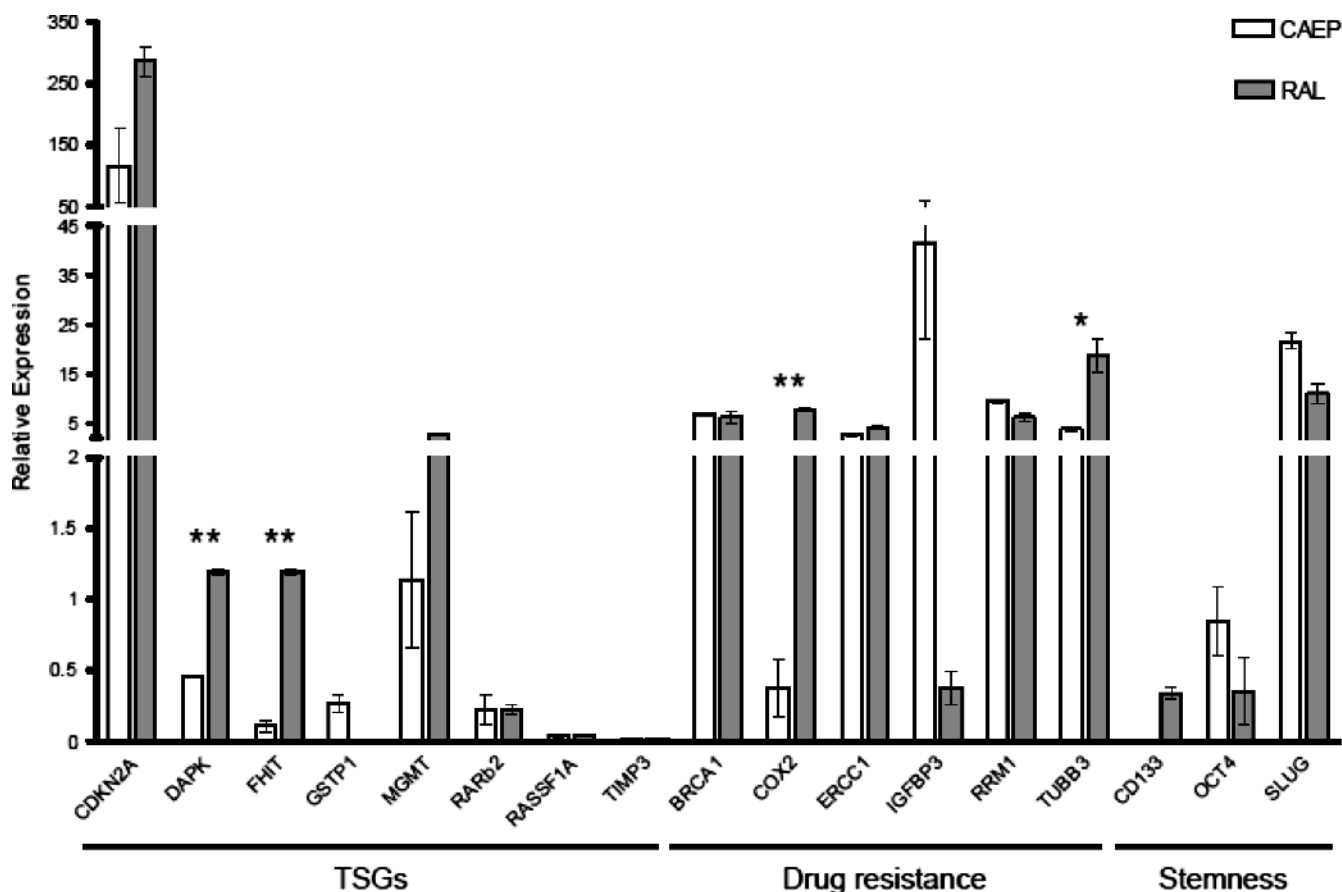
Figure 2. Gene expression profile of CAEP and RAL cell lines in the basal control condition. Expression data were normalized against normal lung tissue. Statistically significant differences are indicated with asterisks, *P < .05; **P < .01.

in CAEP but unmethylated in RAL cells. Genes associated with specific drug resistance (BRCA1, COX2, ERCC1, RRM1, TUBB3) were unmethylated in both cell lines, except for a region of TUBB3 located at 5′ of exon 2 that was partially methylated in CAEP. The promoter of IGFBP3 showed partial methylation in CAEP and no methylation in RAL. When genes associated with stemness features were considered, CD133 and OCT4 were methylated in both cell lines, whereas SLUG was unmethylated (Figures 4 and 5 and Table 1).

Treatment with docetaxel and gemcitabine did not change the above-described methylation profile of our lung cancer cell lines (data not shown).

Discussion

Specific gene expression profiles may be useful for monitoring tumor progression, predicting therapeutic response, and assessing risk of relapse. With the objective of gathering information for clinical purposes,
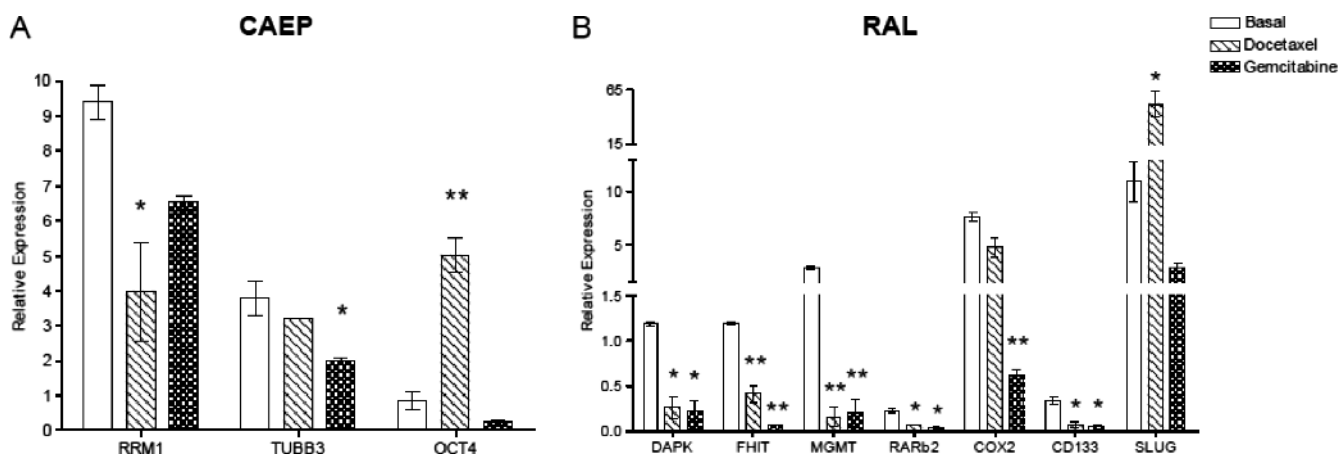

Figure 3. Genes differentially expressed with respect to the basal control condition in (A) CAEP and (B) RAL cell lines after the treatment with docetaxel and gemcitabine. Statistically significant differences are indicated with asterisks, *P < .05; **P < .01.
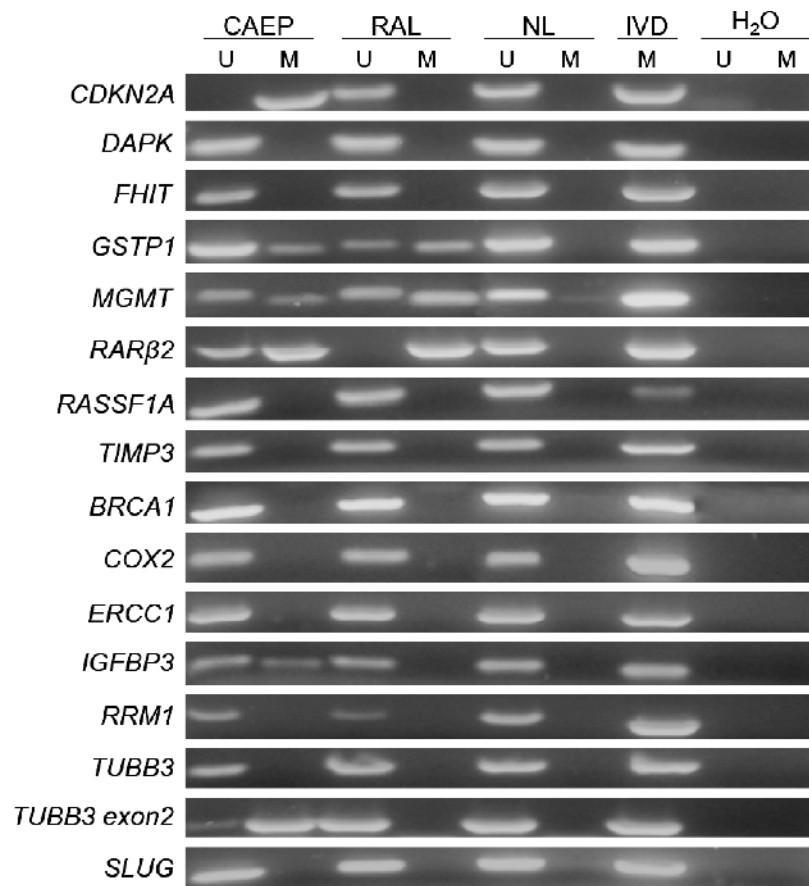
Figure 4. MSP results for CAEP and RAL cell lines in basal control condition. U, unmethylation-specific reaction; M, methylation-specific reaction; NL, normal lymphocyte DNA; IVD, in vitro methylated DNA; H_2O, PCR water control.

in the present work, we used two NSCLC cell lines, representative of a squamous carcinoma (CAEP) and of an adenocarcinoma (RAL), to explore their expression profiles in the basal control condition and after acute treatment with standard lung cancer chemotherapeutic drugs. As was expected considering the neoplastic nature of the cells, both cell lines showed a low expression profile of the TSGs GSTP1, RASSF1A, RARβ2, and TIMP3; DAPK and FHIT were downregulated in only CAEP cells [18,54–57]. Furthermore, genes related to drug resistance were also more widely expressed in both cell lines than in normal lung, as one would predict from the poor efficacy of the standard chemotherapy in the treatment of NSCLCs. Both our cell lines

showed remarkable in vitro resistance to cisplatin, consistent with high basal levels of BRCA1 and ERCC1 mRNAs, as supported by other in vitro and in vivo studies [6,8,58].

However, CAEP and RAL cells differed in their responses to the other drugs investigated, consistent with specific patterns of gene expression. In particular, CAEP cells were less resistant to docetaxel; additionally, in accordance with published data [6–8], they displayed a lower expression level of TUBB3. CAEP cells also showed more sensitivity to gemcitabine, although a plateau in the in vitro chemosensitivity assay was observed in both cell lines. The resistance of both cell lines to plasma peak concentrations of the drug could be

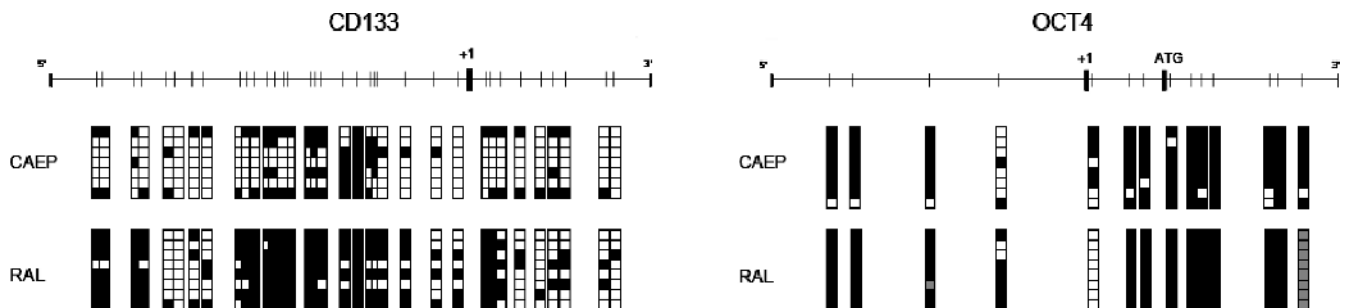**Figure 5.** Bisulfite sequencing results for CD133 and OCT4 genes in CAEP and RAL cell lines in the basal control condition. Vertical bar, CpG site; black square, methylated clone; white square, unmethylated clone; gray square, sequence not defined.

Table 1. DNA Promoter Methylation Status and Gene Expression in CAEP (Squamous Carcinoma) and RAL (Adenocarcinoma) Cell Lines.

Set	Gene Name	CAEP		RAL	
		Methylation	Expression	Methylation	Expression
TSGs	CDKN2A	M	+++	U	+++
	DAPK	U	+	U	++
	FHIT	U	+	U	++
	GSTP1	U/M	+	U/M	–
	MGMT	U/M	++	U/M	++
	RARβ2	U/M	+	M	+
	RASSF1A	U	–	U	–
	TIMP3	U	–	U	–
Drug resistance	BRCA1	U	+++	U	++
	COX2	U	+	U	++
	ERCC1	U	++	U	++
	IGFBP3	U/M	+++	U	+
	RRM1	U	+++	U	+++
	TUBB3	U	++	U	+++
Stemness	CD133	M	–	M	+
	OCT4	M	+	M	+
	SLUG	U	+++	U	+++

U, unmethylated; M, completely methylated; U/M, partially methylated; +++, highly expressed (>5); ++, medium expressed (>1); +, low expressed (<1); –, not expressed.

explained by the observed up-regulation of *RRM1*, a target gene of gemcitabine action [11,59].

The difference in sensitivity to docetaxel and gemcitabine of CAEP *versus* RAL is likely because of different levels of expression of other gene products such as COX2, an important antiapoptotic factor involved in lung carcinogenesis [60] whose expression level was found to be associated with chemoresistance to several agents [4]. Indeed, we found up-regulation of *COX2* gene expression in the adenocarcinoma (RAL) cells, consistent with their greater resistance to all the drugs tested.

The recent findings of small cancer cell populations with stem cell features, i.e., cancer stem cells, in cancers resistant to antitumor agents [46] was the reason for investigating the level of expression of stem-related genes, such as *OCT4*, *CD133*, and *SLUG* [39,43–45] in our cell lines. Indeed, the population surviving docetaxel treatment showed *OCT4* and *SLUG* up-regulation, respectively, in CAEP and RAL cells. This finding highlights the survival of a cell population with stemness features, expected to be more aggressive and chemoresistant according to the literature [37–39,45]. The presence of a resistant component with self-renewing capacity in NSCLC cells is in accordance with the limited survival observed in patients treated with docetaxel as a second-line therapy [61,62].

Because epigenetic mechanisms of gene expression regulation are gaining increasing attention in cancer pathophysiology, we evaluated the methylation status of the promoter of the studied genes. Although promoter methylation is usually associated with gene silencing, we did not find unequivocal evidence of DNA methylation and corresponding low mRNA levels for our entire gene panel. The hypermethylated status of *GSTP1*, *RARβ2*, *CD133*, and *OCT4* promoters correlated with the gene silencing in both cell lines (Table 1). Loss of expression of *GSTP1* and *RARβ2* associated with the hypermethylated status of their promoter regions was also shown by Chen et al. [15], Kerr et al. [27], and Virmani et al. [55]; similarly, the lack of methylation on the promoter region of genes associated with specific drug resistance (*BRCA1*, *COX2*, *ERCC1*, *RRM1*, and *TUBB3*) was in agreement with the detection of their transcripts (Table 1). In addition, *SLUG* was expressed and its promoter was unmethylated, as

expected, in both cell lines (Table 1). Despite *CDKN2A* (in CAEP cells) and *MGMT* gene promoter methylation, as also shown by other authors [15,17,30], these genes were highly expressed in both cell lines (Table 1). The absence of DNA methylation in the promoter of the other genes of interest implies that different mechanism of regulation of gene expression are involved. Genetic alterations, such as allelic loss of chromosome arms, could be also implicated; the 3p arm—harboring *RASSF1A* (3p24), *RARβ2* (3p21.3), and *FHIT* (3p14.2)—is indeed frequently lost in NSCLC [63,64]. The hypermethylated status of *CD133* and *OCT4* promoters is consistent with the differentiated phenotype of our NSCLC cell lines [65,66] and is furthermore correlated with their down-regulation in the basal control condition.

Finally, the treatment with docetaxel and gemcitabine did not cause variations in the promoter methylation status of either cell line. A possible explanation could be the short term of drug treatment, which may not have been sufficient to determine a variation in the methylation profile. Further investigation, which also takes the chromatin structure and histone modifications into account, might better describe the mechanism involved in the modulation of gene expression.

The results reported in this work highlight specific biomarkers consistent with the different responses of the two cell lines to standard pharmacological treatment and indicate a molecular trait of their chemoresistance. CAEP and RAL cell lines may, therefore, be considered validated models for testing novel anticancer compounds. These cell lines may, in addition, represent a simple model for evaluating how cell populations with stem cell features impact cancer resistance to antitumor agents.

Acknowledgments

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